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The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP–ClpX protease, is a novel molecular chaperone

Alicja Wawrzynow[1], Diana Wojtkowiak[1], Jaroslaw Marszalek[1], Bogdan Banecki[1], Matthew Jonsen[2], Barbara Graves[2], Costa Georgopoulos[3] and Maciej Zylicz[1,4]

[1]Division of Biophysics, Department of Molecular Biology, University of Gdansk, 24 Kladki, Gdansk 80-822, Poland, [2]Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, UT 84132, USA and [3]Department of Medical Biochemistry, Centre Médical Universitaire, 1, rue Michel-Servet, 1211 Geneva 4, Switzerland

[4]Corresponding author

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All major classes of protein chaperones, including DnaK (the Hsp70 eukaryotic equivalent) and GroEL (the Hsp60 eukaryotic equivalent) have been found in *Escherichia coli*. Molecular chaperones enhance the yields of correctly folded polypeptides by preventing aggregation and even by disaggregating certain protein aggregates. Previously, we identified the ClpX heat-shock protein of *E.coli* because it enables the ClpP catalytic protease to degrade the bacteriophage λO replication protein. Here we report that ClpX alone possesses all the properties expected of a molecular chaperone protein. Specifically, it can protect the λO protein from heat-induced aggregation, disaggregate preformed λO aggregates, and even promote efficient binding of λO to its DNA recognition sequence. A λO–ClpX specific protein–protein interaction can be detected either by a modified ELISA assay or through the stimulation of ClpX's weak ATPase activity by λO. Unlike the behaviour of the major DnaK and GroEL chaperones, ClpX requires the presence of ATP or its non-hydrolysable analogue ATP-γ-S for efficient interaction with other proteins including the protection of λO from aggregation. However, ClpX's ability to disaggregate λO aggregates requires hydrolysable ATP. We propose that the ClpX protein is a *bona fide* chaperone, whose biological role includes the maintenance of certain polypeptides in a form competent for proteolysis by the ClpP protease. Furthermore, our results suggest that the ClpX protein also performs typical chaperone protein functions independent of ClpP.

Key words: novel chaperone/protease component/protein disaggregation/protein protection/heat-shock protein

Introduction

In recent years, evidence has accumulated for the existence of two classes of heat-shock proteins, molecular chaperones and ATP-dependent proteases. The molecular chaperones, such as the Hsp70 family (the *Escherichia coli* equivalent DnaK) and the Hsp60 family (the *E.coli* equivalent GroEL), have been shown to promote protein folding by mostly inhibiting the non-productive folding events that often lead to protein aggregation. Furthermore, some of the molecular chaperones can protect certain polypeptides from heat inactivation and even disaggregate such aggregates once formed (reviewed in detail in Hendrick and Hartl, 1993 and Georgopoulos and Welch, 1993).

Often the major chaperones do not work in isolation. Rather, they work in synergy with certain other chaperone proteins to constitute 'chaperone machines' (Georgopoulos, 1992). For example, the DnaK, DnaJ and GrpE proteins form one such machine, and GroEL and GroES form another (reviewed in Hendrick and Hartl, 1993 and Georgopoulos and Welch, 1993). Occasionally, there seems to be some overlap in biological action, e.g. both the DnaK and GroEL proteins can protect *E.coli* RNA polymerase from heat inactivation, and both can 'resurrect' RNA polymerase activity from such aggregates, once formed (Skowyra *et al.*, 1990; Ziemienowicz *et al.*, 1993). The major chaperones, GroEL and DnaK, exhibit weak ATPase activities which are usually stimulated by their polypeptide substrates. The binding of ATP alters the conformation of these chaperones, leading to a weakening of the affinity for the substrate, and thus to its release (reviewed in Hendrick and Hartl, 1993; Palleros *et al.*, 1993; Schmid *et al.*, 1994). The DnaJ chaperone, on the other hand, binds to its polypeptide substrates independently of ATP (Gamer *et al.*, 1992; Langer *et al.*, 1992; Liberek and Georgopoulos, 1993). In some situations, the DnaK and GroEL chaperone machines have been shown to associate with both unstable polypeptides and certain bona fide proteases (Sherman and Goldberg, 1991, 1992). This, as well as other *in vivo* studies showing an overall correlation between mutations in any of these chaperone genes and the hypodegradation of aberrant polypeptides, has led to the idea that chaperones may assist protease action by 'presenting' unfolded polypeptides to proteases, or even 'unfolding' certain polypeptides so that they can be more readily digested by proteases (Gross *et al.*, 1990; Parsell *et al.*, 1991; Gottesman and Maurizi, 1992; Squires and Squires, 1992; Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). In other words, if these chaperones cannot 'channel' unfolded intermediates towards a successful folding pathway, they may, in turn, accelerate the rate of degradation by maintaining the intermediates in the unfolded state, thus giving access to the various proteolytic systems.

In both prokaryotes and eukaryotes, the energy-dependent degradation of abnormal proteins or specific unstable proteins can modulate different metabolic pathways, including gene expression, DNA replication, or even the heat-shock response (reviewed in Gottesman and

Maurizi, 1992; Parsell and Lindquist, 1993; Georgopoulos *et al.*, 1994). In *E.coli,* two ATP-dependent protease systems have been characterized in great detail, the Lon (or La) protease and the ClpP–ClpA (or Ti) system (reviewed in Gottesman and Maurizi, 1992). Whereas the Lon protease is a homotetramer, the ClpP–ClpA protease is a large hetero-oligomeric structure composed of six subunits of ClpA and 12 subunits of ClpP (for review see Maurizi, 1992). The 21.5 kDa ClpP subunits form a cylindrical particle, possessing the catalytic core of a serine protease (Maurizi, 1992). By itself, ClpP degrades only short polypeptides in an ATP-independent reaction (Woo *et al.*, 1989). However, larger polypeptides can be hydrolysed if the 83 kDa ClpA subunits are also present (Katajama *et al.*, 1988; Hwang *et al.*, 1988; Thompson and Maurizi, 1994; Thompson *et al.*, 1994). The ClpA protein possesses an ATPase activity stimulated by certain polypeptide substrates (Katajama *et al.*, 1988; Hwang *et al.*, 1988). Furthermore, the binding of ATP is necessary for the assembly of the ClpP–ClpA oligomeric complex and the activation of the ClpP protease (Maurizi, 1992; Thompson and Maurizi, 1994). The overall structure of the ClpP–ClpA protease closely resembles that of the proteosome structure found in yeast (Rechsteiner *et al.*, 1993).

Both the Lon and ClpP–ClpA proteases are responsible for the hydrolysis of abnormal proteins. Since abnormal and denatured proteins accumulate under heat-shock conditions, it was suggested that the Lon and ClpP–ClpA proteases are involved in the degradation of such proteins, thus helping a bacterial cell to survive these adverse biological conditions (Gottesman and Maurizi, 1992).

ClpA belongs to the distinct, highly conserved Hsp100 family of proteins, present in all prokaryotic and eukaryotic organisms thus far tested (Gottesman *et al.*, 1990). The *E.coli* Hsp100 family of proteins is further subdivided into at least four sub-families, A, B, C and X (for review see Parsell and Lindquist, 1993). The ClpA-like proteins contain two ATP binding sites, whose amino acid sequence is highly conserved (Gottesman *et al.*, 1990; Parsell *et al.*, 1991; Squires and Squires, 1992). The *E.coli* ClpB and yeast Hsp100 family members have been purified to homogeneity, and shown to possess a peptide-stimulated ATPase activity (Woo *et al.*, 1992; Parsell *et al.*, 1994). Similarly to ClpA, the yeast Hsp100 was shown to oligomerize in the presence of ATP (Parsell *et al.*, 1994). Neither Hsp100 nor ClpB proteins can substitute for the related ClpA family member in the ClpA-mediated proteolysis of substrates known to be hydrolysed by the ClpP–ClpA protease (Woo *et al.*, 1992). Recently, it was shown that the *Bacillus subtilis* MecB protein (a ClpC family member) is a pleiotropic regulator controlling competence gene expression (Kong and Dubnau, 1994; Msadek *et al.*, 1994).

Recently, we reported the characterization and purification of a new member of the Clp family, ClpX (Wojtkowiak *et al.*, 1993). ClpX was identified because its presence enabled the ClpP catalytic protease to efficiently hydrolyse λO protein, but not other potential substrates, such as α-casein. It turned out that the gene coding for ClpX had already been sequenced, and is the second gene of the *clpP clpX* operon (Gottesman *et al.*, 1993), which is under heat-shock regulation (Kroh and Simon, 1990). The *clpX* gene codes for a truncated member of the Hsp100 family, possessing one ATP binding site as well as a zinc binding motif (Gottesman *et al.*, 1993). The inactivation of the *clpX* gene led to an ~10-fold stabilization of the λO protein *in vivo* (Gottesman *et al.*, 1993). On the basis of such results, it was proposed that ClpX is a 'specificity' factor that targets polypeptide substrates, distinct from those targeted by ClpA, to be hydrolysed by the ClpP catalytic protease (Wojtkowiak *et al.*, 1993; Gottesman *et al.*, 1993). Recently, it has been shown that besides λO, the ClpP–ClpX protease system is capable of degrading the bacteriophage Mu vir repressor (Mhammedi-Alaoui *et al.*, 1994), as well as the PhD protein involved in the stabilization of the P1 plasmid (M.Yarmolinsky, personal communication). Moreover, genetic experiments suggest that ClpX, but not ClpP, is required for normal bacteriophage Mu replication (Mhammedi-Alaoui *et al.*, 1994).

In this work we demonstrate that the ClpX protein can function independently of ClpP, and that it exhibits biological properties associated with molecular chaperones. Specifically, it can protect λO from heat inactivation and disaggregate heat-induced λO aggregates. Furthermore, as with other major chaperone proteins, ClpX's ATPase activity is enhanced by the presence of its λO polypeptide substrate.

Results

ClpX protein protects λO from thermal inactivation

To better understand the molecular mechanism of action of the ClpP–ClpX protease in the bacteriophage λ DNA replication system, we first titrated the ClpX or ClpP components alone, using the previously described *in vitro* λ DNA replication system composed of highly purified proteins (Zylicz *et al.*, 1989). In preliminary experiments, when either purified ClpX or ClpP protein alone was added directly to the replication mixture, no effect on λ DNA synthesis was observed (results not shown). However, during the course of these experiments, we observed that the activity of λO protein is heat labile at 30°C. Thus, when the λO protein was preincubated first at 30°C for 15 min (an equivalent incubation at 45°C had an even more pronounced effect), followed by the addition of the remaining replication proteins, only 5–10% of λ DNA replication activity was observed (Figure 1). Interestingly, the thermal inactivation of λO activity was protected by the addition of the ClpX protein and ATP during the preincubation treatment. As can be seen in Figure 1, increasing amounts of ClpX protein improved the recovery of λO activity. The presence of ATP, during preincubation of λO with ClpX, stimulated the recovery of λO replication activity (Figure 1). When nanomolar concentrations of the ClpX protein were used, the preincubated λO protein was as active as when added directly to the replication mixture at 30°C.

The inactivation of λO was temperature dependent, i.e. preincubation of λO at 0°C with or without ClpX, had no significant effect on λO activity (Figure 1). When either of the well-established DnaK or DnaJ molecular chaperones were added instead of ClpX, protection of λO activity was also observed (Figure 1). The amount of DnaJ or ClpX needed to protect λO was equivalent, whereas for DnaK, at least 20-fold higher levels were required (Figure 1). Furthermore, the λO replication activity was protected

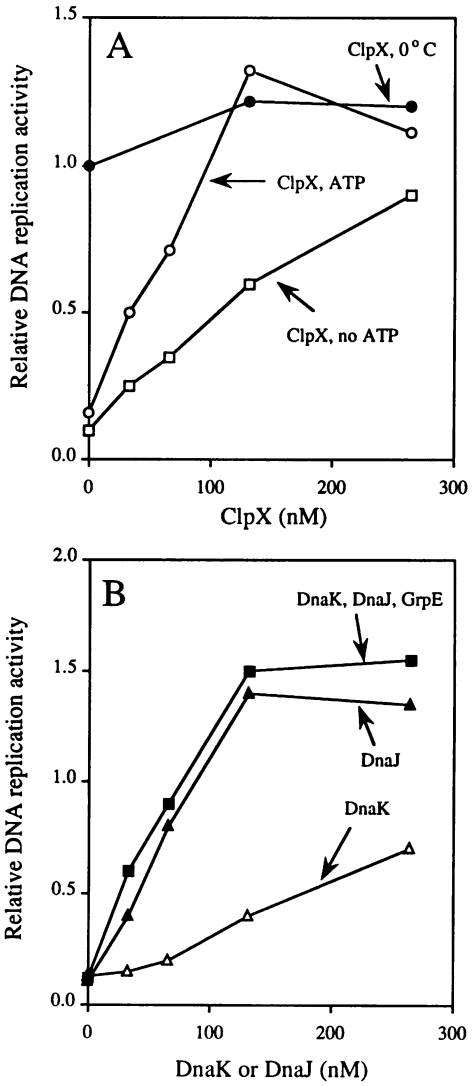

Fig. 1. ClpX, DnaJ and DnaK chaperone proteins can protect λO replication activity from heat inactivation. (**A**) The λO protein (225 nM) was preincubated with the indicated amounts of ClpX, with (○) or without (□) 2 mM ATP in a premixture (12.5 μl) containing: 25 mM HEPES–KOH pH 7.6, 20 mM KCl, 1 mM dithiothreitol (DTT), 7 mM magnesium acetate. Following a 15 min preincubation at 30°C, the replication reaction was initiated by the addition of 12.5 μl of a replication protein mixture containing 300 ng of pRLM4 supercoiled plasmid DNA, 150 ng λP, 150 ng DnaB, 800 ng Ssb, 500 ng DnaK, 50 ng DnaJ, 200 ng GyrA, 90 ng GyrB, 150 ng GrpE, 100 ng DnaG, 200 ng DNA pol III holoenzyme, 100 μM of each dNTP, with [methyl-^{3}H]TTP (50 c.p.m./pmol of dNTP, Amersham), 200 μM each GTP, CTP, UTP, 2.4 mM ATP, 20 μM phosphocreatine, 500 ng creatine kinase and 50 μg/ml bovine serum albumin (BSA) in 40 mM HEPES–KOH pH 7.6, 7 mM magnesium acetate. In a control experiment, ClpX and 2 mM ATP were added to λO and the mixture incubated at 0°C for 15 min (●). (**B**) The λO protein was preincubated with the indicated amount of either DnaK (△) or DnaJ (▲) or various amounts of DnaK, DnaJ (50 μM) and GrpE (100 μM) proteins (■). The remaining replication proteins were added later as part of the replication protein mixture. The amounts of DnaK (500 ng), DnaJ (50 ng) and GrpE (150 ng) were kept constant, unless otherwise indicated. The replication reaction was carried out for 30 min at 30°C, stopped, and the amount of dNMP incorporated into DNA estimated as described by Zylicz *et al.* (1989). Relative DNA replication activity was calculated taking as 100% the value of dNMP incorporated in DNA when λO was kept on ice before the initiation of λ DNA replication (220 pmol). Molar concentrations were calculated assuming that DnaK (70 kDa), DnaJ (40 kDa) and ClpX (46 kDa) are monomeric proteins.

most efficiently when all members of the DnaK chaperone machine, namely DnaK, DnaJ and GrpE, were present during the incubation of λO at high temperature (Figure 1).

ClpX or DnaK enhance λO binding to oriλ

It was previously established that binding of λO to the four repeats of *oriλ* and the subsequent formation of the 'O-some' structure triggers the series of molecular events that lead to λ DNA replication (Dodson *et al.*, 1985; Mensa-Wilmot *et al.*, 1989; Zylicz *et al.*, 1989). It was estimated that more than 100 copies of λO protein are bound per *oriλ* sequence (Dodson *et al.*, 1985). Since the DnaK chaperone was previously shown to stimulate DNA binding proteins to bind their target DNA sequence (Wickner *et al.*, 1991), we compared the ability of DnaK and ClpX to activate λO binding to *oriλ*. To avoid problems involving differential λO binding affinities for these four direct repeat elements, as well as the known cooperativity in λO binding (Tsurimoto and Matsubara, 1981; Roberts and McMacken, 1983), we synthesized the double-stranded (ds) version of one of these direct repeat elements (5′-ATCCCTCAAATTGGGGGAT-3′). This 5′-^{32}P-end-labelled DNA was used to monitor λO binding in gel retardation assays. At low concentrations, the λO protein bound preferentially to the *oriλ* ds DNA sequence, resulting in a well-defined band shift (Figure 2). The exact number of λO molecules bound to each DNA fragment was not estimated. At higher concentrations of λO, additional λO/19-mer ds DNA complexes were formed, as judged by the appearance of a 'smear' during electrophoresis in the 5% polyacrylamide gel (Figure 2A). These protein–DNA complexes are likely non-specific and their different mobilities are most likely due to the varying stoichiometries between λO and 19-mer DNA. At even higher concentrations of λO protein (equivalent to that used in the *in vitro* λ DNA replication assay), most of the λO/19-mer DNA complex did not enter the 5% polyacrylamide gel, most probably due to the formation of non-specific protein–DNA aggregates (Figure 2).

When ClpX protein was preincubated with λO and *oriλ* 19-mer ds DNA, the excessive protein–DNA aggregate formation (as judged by the fraction of DNA which does not enter the 5% polyacrylamide gel) was suppressed, suggesting that ClpX protein prevents λO from aggregating with DNA. In addition, in the presence of ClpX, the amount of specific λO/19-mer ds DNA complex was enhanced, suggesting that the amount of λO protein available for binding to *oriλ* DNA increases. When the ClpX protein was substituted in the reaction by the DnaK chaperone protein, already known to protect other proteins from aggregation (Skowyra *et al.*, 1990; Ziemienowicz *et al.*, 1993), the protein–DNA aggregate formation was suppressed only when DnaK was used at 10- to 100-fold higher concentrations than those used with ClpX. In addition, the recovery of specific λO–DNA complex increased in the presence of DnaK (Figure 2B). We also observed that the presence of the DnaK chaperone decreased the formation of non-specific protein–DNA complexes unable to enter the 5% polyacrylamide gel. Unfortunately, the effect of the DnaJ chaperone on this system could not be tested, based on the fact that it makes non-specific complexes with DNA (Zylicz *et al.*, 1985).

To test whether the ability of ClpX to prevent formation

of non-specific λO–DNA complexes is ATP dependent, we titrated ClpX protein in the presence or absence of ATP. No major effect of ATP was observed (Figure 2C), although we repeatedly were able to show that the presence of ATP always stimulated the recovery of λO–19-mer ds DNA complex.

ClpX, DnaJ or DnaK prevents heat-induced λO aggregation

One interpretation of the ClpX-dependent suppression of non-specific λO–DNA complexes is that ClpX functions as a molecular chaperone to prevent λO aggregation. To directly test this possibility we used sucrose gradient centrifugation to monitor and quantitate the extent of λO aggregation. Such an approach was used successfully by Schröder *et al.* (1993) and Hwang *et al.* (1990) to monitor chaperone action. Under the conditions used for λO binding to *oriλ* 19-mer ds DNA, we found that ~50% of the ^{14}C-labelled λO protein was found in the insoluble (pellet) fraction (result not shown). The proportion of λO protein found in the insoluble fraction increased to 90% when it was preincubated at 45°C for 12 min (Figure 3).

When incubation of λO at 45°C was carried out in the presence of either the DnaJ or DnaK molecular chaperones, the amount of λO protein in the soluble fraction significantly increased (Figure 3A), directly demonstrating their chaperone activity. Protection against λO aggregation by DnaK was reduced in the presence of ATP (Figure 3). This result is consistent with the fact that ATP inhibits a stable DnaK–substrate complex (Liberek *et al.*, 1991b). In contrast, in the presence of ATP, DnaJ's ability to prevent λO aggregation is only slightly affected (Figure 2A). The simultaneous use of DnaK, DnaJ and GrpE did not significantly increase the amount of λO soluble protein (Figure 3A). Previously, we had reported evidence for direct DnaK–λO and DnaJ–λO interactions (Liberek *et al.*, 1990). The results presented here support and extend those earlier findings.

The ClpX-mediated protection of λO in the presence of ATP was as efficient as that provided by DnaJ (Figure 3A and B). However, at least a 20-fold higher concentration of DnaK was needed to accomplish the same level of protection. Unlike DnaK or DnaJ, the ClpX-dependent protection of λO was largely ATP dependent. Either ATP or its non-hydrolysable analogue, ATP-γ-S, was required for efficient protection by ClpX, suggesting that it uses a substrate binding/release mechanism different from that exhibited by the DnaK or GroEL chaperones (Hendrick and Hartl, 1993). Moreover, the presence of *oriλ* ds 19-mer DNA reduced the ATP dependence of this reaction (Figure 3B), thus explaining the fact that we could not detect a dramatic ATP effect in the band-shift assay (Figure 2C).

The ClpX protein can disaggregate λO aggregates

We previously showed that both the DnaK and GroEL chaperone machines are capable of preventing the

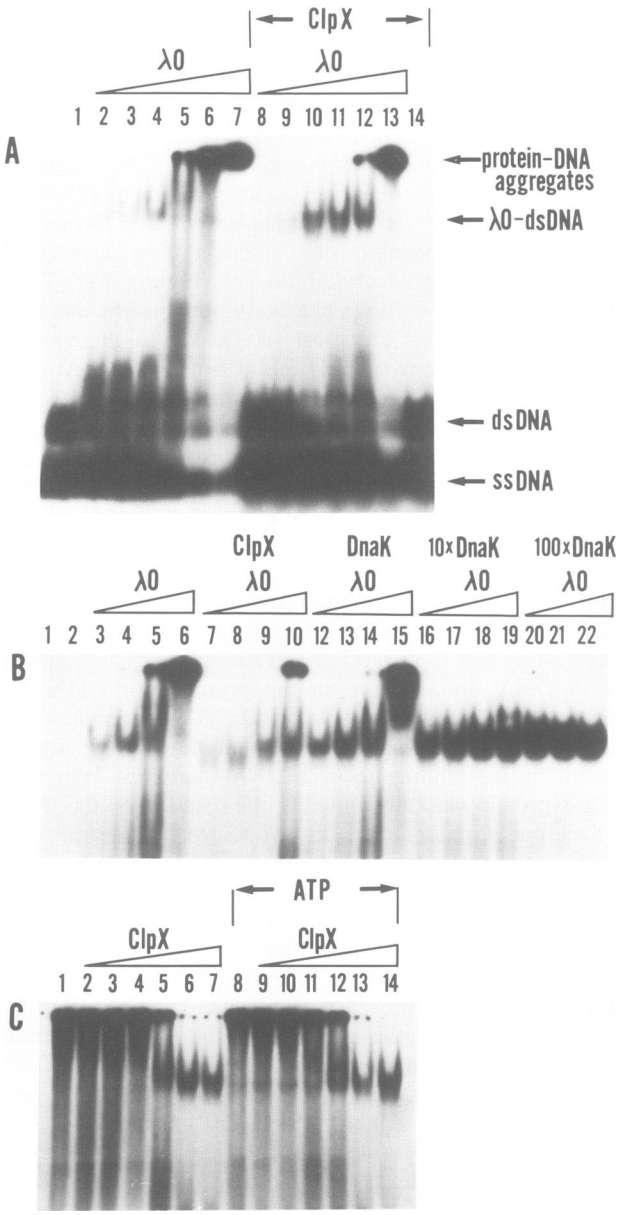

Fig. 2. Binding of λO protein to *oriλ* DNA in the presence of either ClpX or DnaK. (**A**) Increasing amounts of λO protein (lanes 1 and 14, 0 nM; lanes 2 and 8, 22 nM; lanes 3 and 9, 44 nM; lanes 4 and 10, 88 nM; lanes 5 and 11, 176 nM; lanes 6 and 12, 352 nM; lanes 7 and 13, 704 nM) were incubated with 100 pM of ^{32}P-labelled *oriλ* 19-mer ds DNA in a reaction mixture (25 μl) containing: 40 mM HEPES–KOH pH 7.6, 20 mM KCl, 0.5 mg/ml BSA, 2 mM magnesium acetate, 1 mM DTT, and 8% (v/v) glycerol. Lanes 8–14, in addition to λO and *oriλ* 19-mer DNA, contain 260 nM ClpX protein. After 15 min at 30°C the reaction was stopped and electrophoresed on a 5% non-denaturing acrylamide gel as described (Gunther *et al.*, 1990). Molar concentrations were calculated assuming that λO (34 kDa) and ClpX (46 kDa) are monomeric proteins. (**B**) Increasing amounts of λO protein (lanes: 3, 7, 12, 16 and 20, 88 nM; lanes: 4, 8, 13, 17 and 21, 176 nM; lanes: 5, 9, 14, 18 and 22, 352 nM; lanes: 6, 10, 15 and 19, 704 nM) were incubated in the presence of ^{32}P-labelled *oriλ* ,19-mer ds DNA (100 pM) without chaperone (lanes 3–6), with 260 nM of ClpX (lanes 7–10), with 260 nM of DnaK (lanes 12–15), with 2.6 μM of DnaK (lanes 16–19), and with 26 μM of DnaK (lanes 20–22). The experimental conditions were those described in (**A**), except ATP (1 mM) was used. Lane 1 represents a control, where instead of λO, 260 nM ClpX was added. Lane 2 represents a control, where instead of λO, 2.6 μM DnaK was added. (**C**) λO protein (260 nM) was preincubated with ^{32}P-labelled *oriλ* 19-mer ds DNA in the presence of increasing amounts of ClpX protein: lanes 1 and 8, 0 M; lanes 2 and 9, 13 nM; lanes 3 and 10, 26 nM; lanes 4 and 11, 74 nM; lanes 5 and 12, 150 nM; lanes 6 and 13, 260 nM; lanes 7 and 14, 390 nM in the absence (lanes 1–7) and presence (lanes 8–14) of 1 mM ATP. The assay conditions were the same as those described in (A).

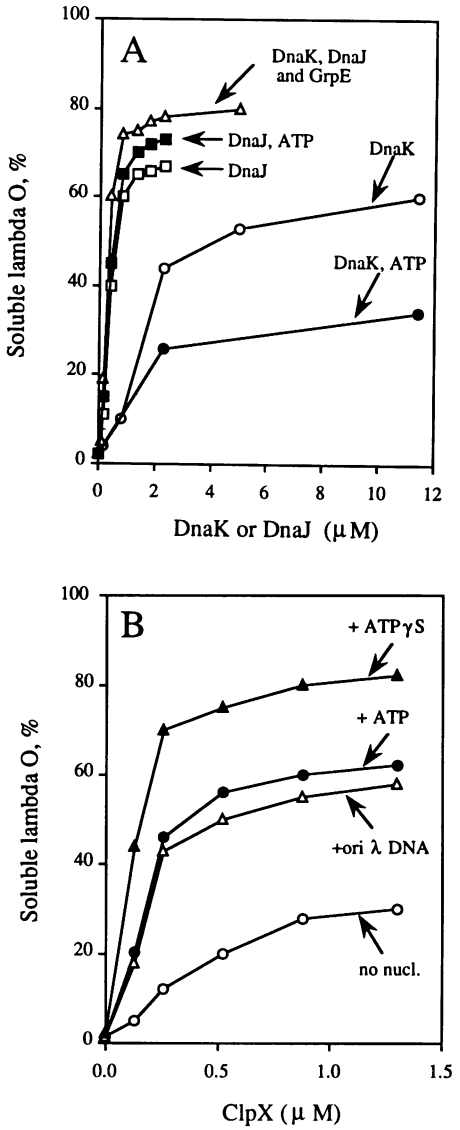

Fig. 3. ATP or ATP-γ-S stimulates ClpX-dependent protection of λO from aggregation. (**A**) ^{14}C-labelled λO protein (260 nM) was preincubated for 5 min at 30°C under the buffer conditions described in the legend to Figure 2A with increasing amounts of various molecular chaperones and then transferred to 45°C for an additional 12 min. Following this, the reaction mixture (25 μl) was layered over a 50 μl 20% (w/v) sucrose cushion, preformed in a 0.5 ml microfuge tube. The buffer conditions of the sucrose solution were the same as those of the reaction mixture. When the reaction mixture included phosphonucleotides, the same concentration of phosphonucleotides was included in the sucrose solution. Following a 20 min centrifugation (40 000 *g*, 4°C, Beckman JA-20 rotor) 50 μl of liquid from the top of the tube were collected (soluble fraction) and transferred directly to scintillation fluid. Following this, 25 μl of 0.1% (w/v) SDS were added to the microfuge tube. The tube was extensively vortexed before transferring the liquid to scintillation fluid (non-soluble fraction). The overall recovery of λO protein was >90% (soluble + non-soluble fraction). The value of 100% was the amount of λO protein recovered from a 25 μl reaction mixture that was incubated on ice and directly transferred (without centrifugation) to the scintillation cocktail. The experiments were repeated three times, and each experimental determination was in duplicate. The average number of all six experimental determinations is shown. Experimental error was not more than 10% of the value shown. O, DnaK alone; ●, DnaK with ATP (1 mM); △, increasing amounts of DnaK in the presence of 0.12 μM DnaJ, 2 μM GrpE and 1 mM ATP; □, DnaJ alone; ■, DnaJ with ATP (1 mM). (**B**) The experiment was performed as described in (A) except that ClpX was used instead of the DnaJ or DnaK molecular chaperones. O, ClpX alone; ●, ClpX with ATP (1 mM); ▲, ClpX with ATP-γ-S (1 mM); △, ClpX with *ori*λ 19-mer ds DNA (10 μM)

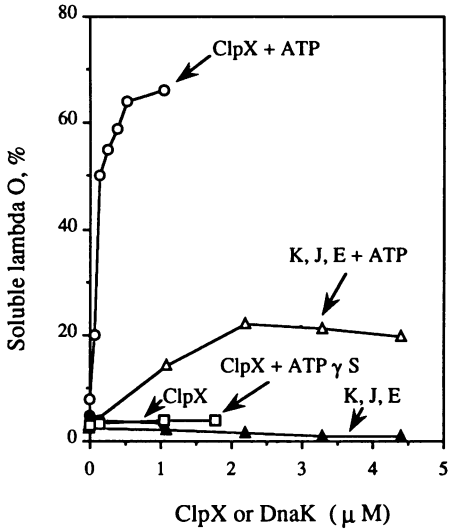

Fig. 4. ClpX can dissolve λO aggregates in an ATP-dependent reaction. ^{14}C-labelled λO protein (260 nM) was preincubated for 5 min at 30°C and 12 min at 45°C without any chaperone proteins under the buffer conditions described in Figure 2A. Following the incubation at 45°C, the reaction mixture was cooled to 30°C in the presence of increasing amounts of ClpX, with ATP (O), with ATP-γ-S (□) or without any nucleotide (●). The reaction proceeded for an additional 1 h. The amount of ^{14}C-labelled λO protein in the soluble fraction was estimated using sucrose gradient centrifugation as described in Figure 3A. In a control experiment, instead of ClpX, a mixture containing increasing amounts of DnaK protein in the presence of DnaJ (0.12 μM) and GrpE (2 μM), with (△) or without (▲) ATP was added to the heat-inactivated λO protein. The incubation proceeded for 1 h at 30°C.

formation of and disaggregating *E.coli* RNA polymerase heat-induced aggregates once formed (Skowyra *et al.*, 1990; Ziemienowicz *et al.*, 1993). We asked whether ClpX protein was also capable of disaggregating λO aggregates, by employing sucrose gradient centrifugation. As shown above, when the λO protein was first preincubated for 12 min at 45°C, at least 90% of it was found in the form of aggregates (pellet fraction) (Figure 4). When this aggregated λO preparation was incubated with ClpX protein and ATP for 60 min at 30°C, more than 60% of the λO protein was disaggregated and sedimented in the soluble fraction (Figure 4). The ClpX-promoted λO disaggregation reaction was completely ATP dependent and was not observed when the non-hydrolysable analogue ATP-γ-S was used (Figure 4). The titration of ClpX protein showed that the disaggregation of λO occurred at stoichiometric levels (Figure 4). In contrast, the DnaK/DnaJ/GrpE chaperone system was much less efficient in this case (Figure 4). Yet, this amount of DnaK/DnaJ/GrpE was previously shown to be capable of completely disaggregating heat-induced RNAP aggregates (Ziemienowicz *et al.*, 1993 and result not shown). In order to confirm our results with the sucrose gradient centrifugation technique, we also performed size exclusion chromatography. Following heat inactivation, most of the ^{14}C-labelled λO protein eluted in the void volume of a Sepharose 4B column (Figure 5). The presence of ClpX during the heat inactivation step significantly reduced λO aggregate formation (Figure 5), thus confirming our results obtained with the sucrose gradient centrifugation technique (see Figure 3). Moreover, when λO protein was first heat

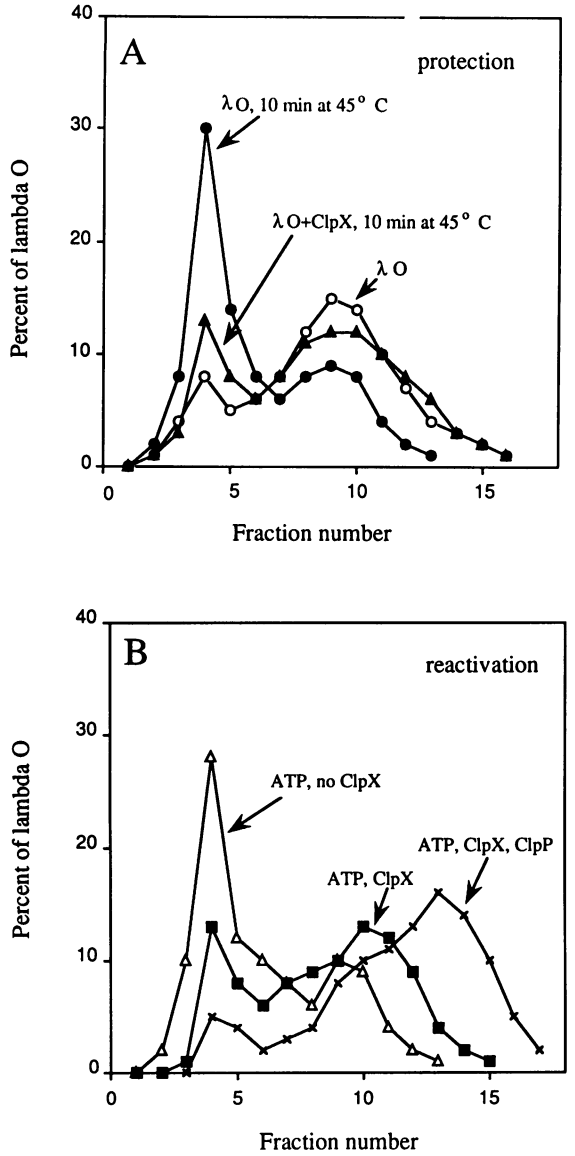

Fig. 5. Size exclusion chromatography of ^{14}C-labelled λO protein in the presence of ClpX chaperone. (**A**) ^{14}C-labelled λO protein (260 μM) was preincubated first at 30°C for 5 min and then at 45°C for 10 min with (▲) or without (●) ClpX (1 μM) protein in reaction mixtures containing 40 mM HEPES–KOH pH 7.6, 100 mM KCl, 0.5 mM BSA, 2 mM magnesium acetate, 1 mM DTT and 1 mM ATP. The reaction mixture (50 μl) was loaded onto a Sepharose 4B column as described by Wojtkowiak *et al.* (1993). The amount of ^{14}C-labelled λO protein in each column fraction was detected by liquid scintillation counting. (**B**) Following heat inactivation of the ^{14}C-labelled λO protein, under the experimental conditions described in (A), the reaction mixture was incubated at 30°C for 60 min with 1 ATP (△) or with 1 mM ATP, ClpX (1 μM) (■) or with 1 mM ATP, ClpX (1 μM) and ClpP (1 μM) (x). Size exclusion chromatography was performed as described in (A).

inactivated in the absence of ClpX, and subsequently treated with ClpX and ATP, a substantial amount of λO aggregates were dissolved, as judged by the appearance of dimeric λO protein in the included volume (Figure 5). These results also confirm that during the disaggregation assay the ClpX protein did not degrade the λO protein. Figure 5 shows that λO proteolysis requires, in addition to ClpX and ATP, the simultaneous presence of the ClpP catalytic component, as judged by the position of the ^{14}C-labelled material in the included volume (Figure 5).

ClpX binds directly to λO

One possible explanation of why ATP is needed for both the protection from aggregation and the reactivation of λO protein aggregates is that ATP is important for the binding of ClpX to its substrate, λO. We previously showed that the ClpX protein can promote the ClpP-dependent proteolysis of λO only in the presence of ATP (Wojtkowiak *et al.*, 1993). In this reaction, ATP could not be substituted by its non-hydrolysable analogue ATP-γ-S (result not shown).

In order to monitor in a more sensitive way the protein–protein interaction between ClpX and λO, we used a modified ELISA assay described by Marszalek and Kaguni (1994). The λO protein was first allowed to fix in the wells of an ELISA plate, followed by blocking of the rest of the binding sites with excess BSA. The ClpX protein was then added in the presence of BSA and 0.05% Triton X-100 to prevent non-specific protein–protein interactions. Following washing, the amount of ClpX bound to λO was detected by first incubating with rabbit anti-ClpX antibodies, followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase. As suggested by the previously observed protection of λO from heat-induced aggregation by ClpX (see sucrose gradient experiments above; Figure 3), the affinity of ClpX for λO was stimulated in the presence of either ATP or ATP-γ-S, as quantitated by this modified ELISA assay (Figure 6A). To verify that ClpX binding to λO is substrate specific, we tested ClpX's affinity for other potential protein substrates. Using ATP-γ-S (optimal for λO–ClpX interaction), we could not detect substantial ClpX binding to either the DnaK chaperone protein or BSA (Figure 6B). Furthermore, both the σ^{32} and λP proteins bound much more weakly than λO to ClpX (Figure 6B), suggesting that λO possesses either a specific amino acid sequence or a conformation that is specifically recognized by ClpX.

Consistent with these results, in separate experiments, we showed that the ClpP–ClpX protease system did not appreciably degrade either λP, σ^{32} or DnaK (Figure 7). Using our standard assay conditions for ClpX-dependent proteolysis of λO (Wojtkowiak *et al.*, 1993), we showed that only 2% of λP, 1% of σ^{32} α-casein DnaK proteins were hydrolysed, whereas 98% of λO was hydrolysed, under similar conditions. Such hydrolysis required the presence of ATP, since substitution by ATP-γ-S did not promote substantial λO proteolysis. The substitution of ClpX by ClpA changed the ability of the ClpP protease to hydrolyse different protein substrates. For example, the ClpA–ClpP proteolytic system degraded λO, albeit to a limited extent, did not degrade DnaK at all, but did degrade σ^{32} and α-casein to an appreciable extent (Figure 7). These results support the idea that ClpA and ClpX behave as protein specificity factors, by presenting different polypeptide substrates to the ClpP catalytic protease (Wojtkowiak *et al.*, 1993; Gottesman *et al.*, 1993). The biological significance of the ClpA–ClpP proteolysis of σ^{32}, if any, remains to be addressed.

We were unsuccessful in our attempts to detect the formation of a soluble λO–ClpX protein complex, using size exclusion chromatography, suggesting that the λO–ClpX interaction is either transient or that it fortuitously has the same size as ClpX (perhaps through a reduction in the oligomeric state of ClpX in the λO–ClpX complex).

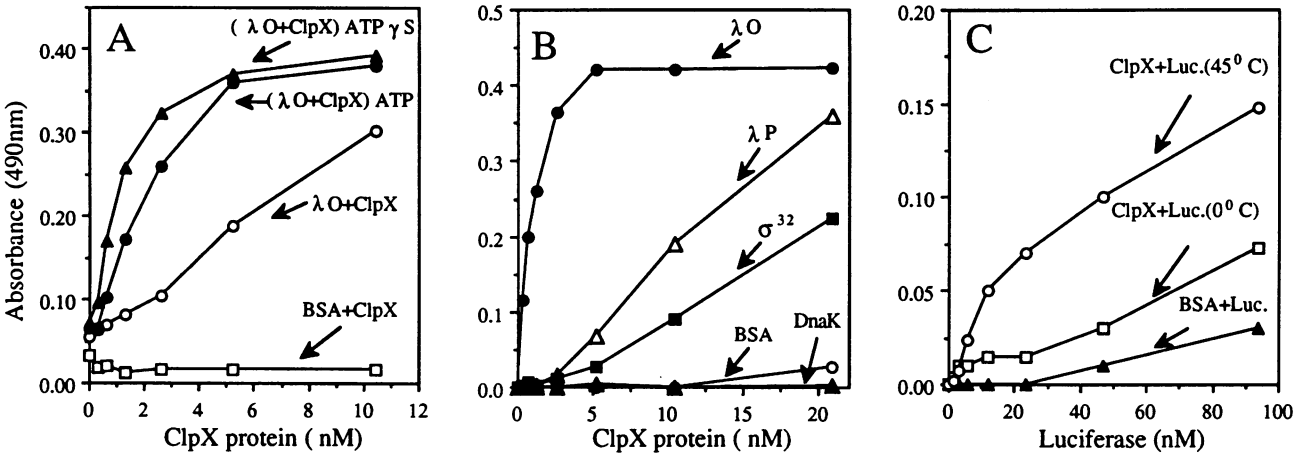

Fig. 6. Binding of ClpX to various protein substrates. (**A**) λO protein or BSA (50 µl, 0.01 mg/ml) in PBS buffer (0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4) was incubated for 1 h at room temperature in each well of an ELISA plate. After decanting the liquid, the wells were washed twice with 100 µl of buffer A (0.2% BSA in PBS) and incubated with 200 µl of buffer A for an additional 1 h. After decanting the liquid, the wells were washed once with buffer B (25 mM HEPES–KOH pH 7.6, 150 mM KCl, 25 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 2.5% (v/v) glycerol, 0.1 mM EDTA, 0.05% Triton X-100 and 0.2% BSA). Following this, increasing amounts of ClpX protein in buffer B (50 µl) were added with or without phosphonucleotides and the total volume of the reaction was adjusted to 60 µl with buffer B. After incubating for 30 min at room temperature, the ELISA plate was washed once with 100 µl of buffer B and three times with 100 µl of buffer A. Following this, 100 µl of a 1:10 000 dilution (in buffer A) of anti-ClpX serum was added and the plates incubated for 2 h at room temperature. After washing three times with 100 µl of buffer A, 100 µl of a 1:3000 dilution (in buffer A) of goat anti-rabbit IgG (H+L) serum conjugated to horseradish peroxidase (Bio-Rad) was added and the incubation proceeded for an additional 45 min. The wells were washed three times with 100 µl of buffer A and a colour assay using the TMB peroxidase EIA substrate kit (Bio-Rad) was performed. The absorbance (490 nm) was estimated using a Termo Max microplate reader (Molecular Devices). □, BSA in the well, ClpX added as the second protein; O, λO in the well, ClpX added as the second protein;●, λO in the well, ClpX added as the second protein in the presence of 1 mM ATP; ▲, λO in the well, ClpX added as the second protein in the presence of 1 mM ATP-γ-S. (**B**) λO (●), λP (△), σ^{32} (■), DnaK (▲) or BSA (O) were added to the ELISA plate wells (50 µl of 0.01 mg/ml protein solutions in PBS) and incubated for 1 h at room temperature. Following the washing and blocking procedures, described in (A), increasing amounts of ClpX protein in buffer B were added and the amount of ClpX bound to different substrates was estimated as described in (A). (**C**) ClpX (50 µl of 0.01 mg/ml solution in PBS) was incubated in the wells for 1 h at room temperature. Following the washing and blocking procedures described above, increasing amounts of heat-treated (○) or untreated, i.e. kept at 0°C (□) firefly luciferase were added in buffer B. The amount of luciferase bound to BSA or ClpX was detected using a 1:10 000 dilution of anti-luciferase serum using the procedure described in (A). Before loading the wells, luciferase (5 mg/ml stock solution) was gently diluted 100 times in PBS buffer and incubated at 0°C or 45°C for 12 min. Following that incubation, luciferase was diluted with buffer B to the desired concentration and added to the wells. The symbol (▲) represents heat-treated luciferase bound to BSA. All experimental points represent the average value of six experimental determinations, the estimated error being <5% of the value shown.

It is known that most molecular chaperones recognize the denatured form better than the native form of most of their protein substrates (reviewed in Hendrick and Hartl, 1993). To see whether this is also true for ClpX, we tested its ability to bind to native or denatured (heat-treated) firefly luciferase (Figure 6C). It has been previously shown by others that incubation of luciferase at 42°C for 10 min results in its denaturation (Schröder *et al.*, 1993). We found that ClpX binds to denatured luciferase better than to native luciferase (Figure 6C), although the overall affinity of ClpX for denatured luciferase is much lower than that for λO (Figure 6B and C). Consistent with this, ClpX could not reactivate heat inactivated luciferase, under conditions optimal for the DnaK/DnaJ/GrpE system (Schröder *et al.*, 1993).

The λO protein stimulates ClpX's ATPase activity

Previous studies have established that bona fide chaperones such as Hsp70 (DnaK) and Hsp60 (GroEL) exhibit an accelerated ATPase activity in the presence of the appropriate polypeptide substrates (reviewed in Hendrick and Hartl, 1993). As shown in Figure 8, highly purified ClpX protein alone possesses a weak ATPase activity whose K_m is ~500 µM. This weak ATPase activity is specifically stimulated by λO, but not BSA (Figure 8A). The λO stimulatory effect is due to an increase in ClpX's V_{max} (~3-fold), and not to a significant change in its affinity for ATP, as judged by the unaltered K_m value (Figure 8B). These results are consistent with the behaviour of other members of the Hsp100 family, e.g. purified ClpB protein's ATPase activity is stimulated in the presence of casein (Woo *et al.*, 1992).

Discussion

Over the past few years, work from many laboratories has uncovered the existence of various molecular chaperone machines (reviewed in Georgopoulos, 1992; Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993). The major chaperone machines, such as DnaK and GroEL, have been conserved throughout evolution. Their primary role is to assist correct polypeptide folding by minimizing protein aggregation, and disaggregate various protein aggregates once formed. All of the individual components that make up the DnaK and GroEL chaperone machines belong to the family of so-called heat-shock or stress proteins, whose expression is induced upon a shift-up in temperature or under conditions of stress (reviewed in Yura *et al.*, 1993). The reason for this is that protein aggregation is favoured at higher temperature, primarily due to increased protein unfolding on the one hand, and to the increased importance of hydrophobic interactions on the other.

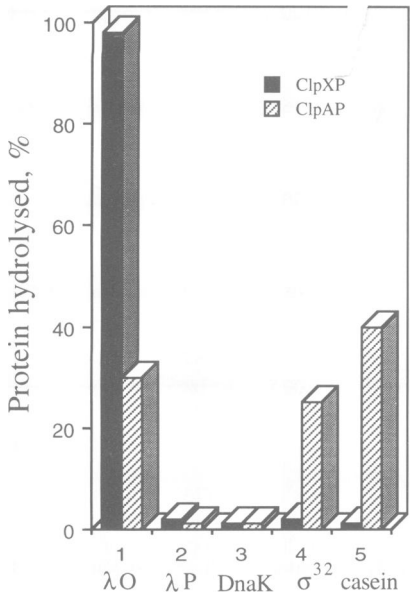

Fig. 7. ClpX- or ClpA-dependent proteolysis of different protein substrates. ClpX (0.5 pmol) or ClpA (0.5 pmol) was incubated with different ^{14}C-labelled protein substrates (5 pmol each) in the presence of 10 pmol ClpP. The reaction mixture (100 µl), containing 20 mM HEPES−KOH pH 7.2, 10 mM magnesium acetate, 10 mM ATP, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.5% Brij 58, was incubated at 30°C for 40 min. The reaction was stopped by the addition of ice-cold trichloroacetic acid to 10% (w/v) and the amount of acid-soluble radioactivity estimated or described by Wojtkowiak *et al.* (1993).

Pelham (1986) originally proposed that molecular chaperones may help the recovery from heat through their ability to prevent protein aggregation and by promoting disaggregation. A few years later, it was shown in a purified system that the DnaK chaperone can protect *E.coli* RNA polymerase from heat-induced aggregation in an ATP-independent reaction, as well as disaggregate thus-formed aggregates in an ATP-dependent reaction (Skowyra *et al.*, 1990). Subsequently, it was shown that the GroEL chaperone machine can also promote these protection and disaggregation reactions (Ziemienowicz *et al.*, 1993). The recovery process must also include the action of proteases in order to remove heavily denatured proteins, perhaps those which cannot be disaggregated by chaperones (Gottesman and Maurizi, 1992). In analogy with the eukaryotic ubiquitin system, the bacterial DnaK chaperone system may specifically 'tag' some denatured proteins and thus promote their efficient degradation (Sherman and Goldberg, 1992).

The possible involvement of chaperone proteins in proteolysis was directly raised by Squires and Squires (1992) in the case of Hsp100 heat-shock family members. Such a possibility was supported by both genetic and biochemical data, namely (i) ClpA, the ATPase component of the ClpP−ClpA protease system, could bind the substrate and, in an ATP-promoted reaction, make it more accessible for ClpP-dependent proteolysis, perhaps by partially unfolding it (Katayama *et al.*, 1988; Parsell *et al.*, 1991; Thompson and Maurizi, 1994; Thompson *et al.*, 1994), and (ii) genetic experiments had shown that the thermotolerance loss caused by a mutation in the yeast Hsp100 gene (homologue of the *E.coli clpB* gene) could be reversed through the overproduction of Hsp70 (Sanchez

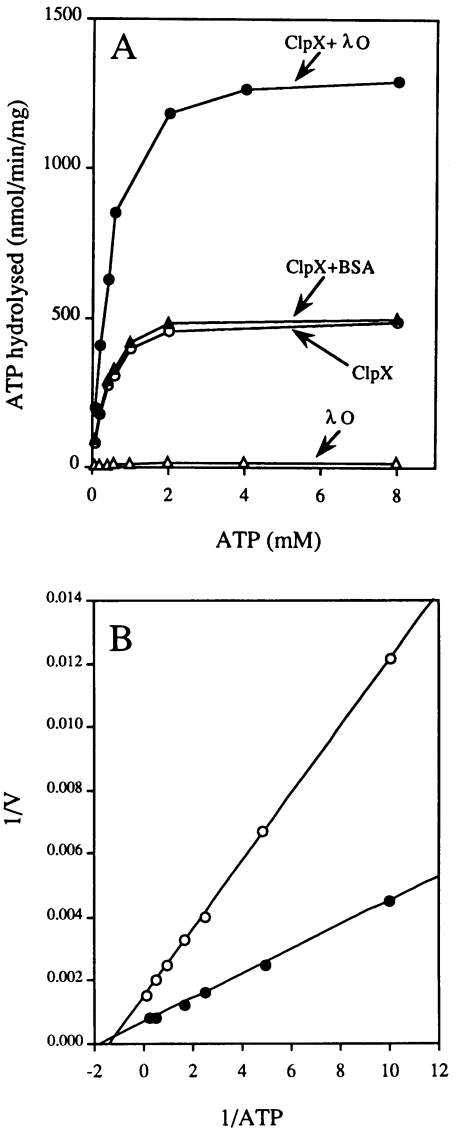

Fig. 8. The λO protein stimulates ClpX's ATPase activity. (**A**) ClpX protein (130 nM) was incubated with (●) or without (○) λO protein (220 nM) or BSA (500 nM) (▲) in a reaction mixture (25 µl) containing: 25 mM HEPES−KOH pH 7.6, 5 mM magnesium acetate, 100 mM KCl, 1 mM DTT and 0.75 µl of [^{3}H]ATP (1 mCi/ml, Amersham) with different concentrations of unlabelled ATP. The kinetics of ATP hydrolysis were estimated as previously described (Liberek *et al.*, 1991a). In a control experiment, ATP hydrolysed in the presence of λO protein alone (220 nM) was determined (△). The K_m for the ClpX protein alone was estimated at 500 ± 50 µM. The K_m for the ClpX in the presence of λO protein was estimated to be 550 ± 50 µM. (**B**) The same experimental data shown in (A) are plotted in a different form. As can be seen, the estimated K_m value (550 ± 50 µM) of ClpX's ATPase activity does not change substantially in the presence of λO.

et al., 1993). Conversely, the yeast Hsp100 protein was shown to be important for cell growth, under limiting Hsp70 conditions (Sanchez *et al.*, 1993).

Our previous work led to the identification of a new component for the ClpP protease, namely ClpX. The 46 kDa ClpX protein was purified as part of the ClpP−ClpX bipartite protease system, based on its ability to degrade native bacteriophage λO protein in an ATP-dependent reaction (Wojtkowiak *et al.*, 1993). The ClpX protein was shown to be a specificity factor because it promoted the

ClpP-dependent degradation of λO but not that of α-casein. Substitution of ClpX by the previously identified ClpA protein partially suppressed λO degradation and instead promoted efficient hydrolysis of α-casein. When the N-terminal sequence of ClpX protein was determined, it became apparent that the corresponding *clpX* gene had already been cloned and sequenced as the distal gene of the *clpP clpX* heat-shock operon (Gottesman *et al.*, 1993). The *in vivo* half-life of λO was stabilized ~10-fold in either a *clpP* or *clpX* null mutant background, demonstrating that the ClpP−ClpX proteolytic system is responsible for most of the λO intracellular degradation (Gottesman *et al.*, 1993). Protein sequence analysis showed that ClpX possessed a substantial degree of homology to previously identified chaperone proteins involved in secretion, namely cdc48 and valosin-containing protein (Gottesman *et al.*, 1993).

In this paper we present direct evidence demonstrating that ClpX can indeed perform chaperone functions in the absence of the ClpP catalytic protease. In a nutshell, our evidence consists of the following: (1) ClpX can protect λO protein from heat-induced aggregation. In so doing, ClpX enhances binding of λO to its *oriλ* ds DNA sequence, thus promoting efficient λ DNA replication. A similar protection of λO from heat inactivation and enhancement of *oriλ* DNA binding can be observed with bona fide chaperones such as DnaJ or DnaK. Interestingly, heat protection of λO by ClpX was stimulated in the presence of either ATP or its non-hydrolysable analogue ATP-γ-S; (2) the ClpX protein efficiently disaggregated heat-induced λO aggregates. Since the native molecular weight of ClpX has not been determined, it is not yet known whether it works more efficiently than the DnaK/DnaJ/GrpE system in disaggregating λO aggregates. For the λO disaggregation reaction, the presence of ATP was required, its ATP-γ-S non-hydrolysable analogue being mostly ineffective. In both the *in vivo* protection and 'resurrection' (disaggregation) reactions, the ClpX chaperone can work in the absence of any other protein components. Since almost stoichiometric amounts of the ClpX chaperone are needed to dissolve λO aggregates, it could be that ClpX acts on aggregation-sensitive unfolding intermediates.

Using a sensitive ELISA assay, we were able to demonstrate a specific protein−protein interaction between ClpX and λO. The binding of ClpX to λO is rather specific since other tested proteins bind less efficiently to the ClpX chaperone than λO. In agreement with the heat protection experiment, either ATP or ATP-γ-S stimulated this protein−protein interaction. In the case of the DnaK chaperone, Schmid *et al.* (1994) showed that ATP binding accelerates 47-fold the rate of substrate binding, but it also accelerates 440-fold the rate of substrate release, thus exerting an overall negative effect on the DnaK–substrate interaction. Obviously, the case with ClpX must be radically different, since ATP has an overall stabilizing effect on the λO–ClpX interaction. The ELISA test showed that the ClpX protein behaved like a chaperone, inasmuch as it interacted better with heat-aggregated luciferase than with the unheated control. The idea emerging from our work and that of others is that the various members of the Hsp100 family, namely ClpA, ClpB and ClpX, may present a different spectrum of protein substrates to ClpP or other catalytic proteases. The use of a particular Hsp100 member will depend on the strength of its relative binding to a specific protein substrate. In the system described here, ClpA will preferentially present α-casein to ClpP, whereas ClpX will preferentially present λO to ClpP. Most likely, some polypeptide substrates can be effectively presented by more than one Hsp100 member or other bona fide chaperones. It remains to be seen whether the various Hsp100 members perform solitary tasks *in vivo*, such as the protection and disaggregation of other polypeptides, or whether they always work synergistically with catalytic proteases. For example, it was recently shown by Mhammedi-Alaoui *et al.* (1994) that ClpX, but not ClpP, is needed for *in vivo* phage Mu DNA replication, indicating that ClpX performs biological functions in the absence of ClpP.

While protection of λO from aggregation by ClpX and binding of λO to ClpX protein occur in the presence of either ATP or the analogue ATP-γ-S, disaggregation of λO aggregates by ClpX occurs only with ATP. This ATP requirement may reflect the need for ClpX to undergo many cycles of conformational changes, in order to efficiently 'massage' its λO-aggregated substrate, perhaps leading to a partial unfolding of the λO protein.

The possibility of 'unfoldase' activity by the Hsp100 family of proteins was suggested before by Squires and Squires (1992), Katayama *et al.* (1988) and Parsell *et al.* (1991). Recent work performed in M.Maurizi's laboratory (Thompson and Maurizi, 1994; Thompson *et al.*, 1994) showed that binding of ATP-γ-S to ClpA promotes not only the oligomerization of ClpA, but also the binding of ClpA to the ClpP proteolytic subunit, thus activating ClpP. The ATP-γ-S-activated ClpP−ClpA protease could hydrolyse some, but not all, of its substrates in a processive manner. However, ATP hydrolysis was absolutely required for the processive proteolysis of α-casein. The authors speculate that the ATP hydrolysis step is not important for the processivity of protease action, but rather is involved in the initial steps of capturing and presenting the substrate to the ClpP protease subunit. Similar to the hydrolysis of α-casein by ClpA, hydrolysis of λO promoted by the ClpP−ClpX protease absolutely requires ATP. In analogy with ClpP−ClpA, ATP-γ-S also served for binding of λO to ClpX (this paper) and λO to ClpP via ClpX (unpublished results).

Recently, it has been shown that the ClpA protein can substitute for the DnaK/DnaJ/GrpE chaperone machine in the activation of the bacteriophage P1 RepA protein, allowing RepA to bind to its *ori* DNA sequence (S.Wickner and K.McKenney, personal communication). The same group had previously shown that this activation step involves the monomerization of RepA dimers (Wickner *et al.*, 1991). Thus, it appears that, in addition to ClpX, other members of the Hsp100 family of proteins perform similar protein disaggregation functions.

Materials and methods

Proteins

In all experiments described in the text, highly purified proteins (90% or greater purity) were used. The ClpX and ClpP proteins were purified essentially as described by Wojtkowiak *et al.* (1993), with modifications that will be described elsewhere. The σ^{32} and ^{35}S-labelled σ^{32} proteins were purified as described by Liberek *et al.* (1992). DnaK was purified as described by Zylicz and Georgopoulos (1984), with the modification

described by Zylicz *et al.* (1989). DnaJ was purified as described by Zylicz *et al.* (1985), and GrpE was purified as described by Zylicz *et al.* (1987). The λO, λP and the *E.coli* replication proteins were purified as described by Zylicz *et al.* (1989). The ^{14}C-labelled λO protein, DnaK and λP were purified as described by Liberek *et al.* (1988) and Wojtkowiak *et al.* (1993). Firefly luciferase was purchased from Sigma. Rabbit anti-luciferase serum was purchased from East Acres Biologicals. Rabbit anti-ClpX and anti-ClpA sera were the generous gift of Dr. M.Maurizi (NIH).

DNAs

The oligonucleotides: 5′-ATCCCTCAAATTGGGGGAT-3′ and 5′-ATCCCCCAATTTGAGGGAT-3′ were synthesized by Dr Robert Schackman (University of Utah Core Facility). The concentration of the DNA strands was estimated spectrophotometrically. The molar absorption coefficient was determined from the nucleotide sequence using the Oligo 4.0 program. The two strands were gel-purified (Maniatis *et al.*, 1982) and end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The strands were mixed in equimolar concentrations and boiled for 3 min, followed by slow cooling (2 h). Unincorporated [γ-^{32}P]ATP was removed using a Bio-Rad spin column.

DNA replication

The *in vitro* purified DNA replication system was as previously described by Zylicz *et al.* (1989).

Band shift assay

The band shift technique for detecting the formation of λO/*ori*λ 19-mer ds DNA complexes was performed as previously described by Gunther *et al.* (1990).

Sucrose gradient centrifugation

Estimation of the soluble fraction of λO protein was a modified version of that described by Hwang *et al.* (1990). See Figure legends for more details.

Protein–protein interaction assay

The ELISA assay used to detect protein–protein interactions was a modified version of that previously published by Marszalek and Kaguni (1994). See Figure legends for more details. The ClpX ATPase assay was carried out as previously described by Liberek *et al.* (1991a).

Proteolysis assay

The proteolysis assays were performed as described previously by Wojtkowiak *et al.* (1993), as was size exclusion chromatography of ^{14}C-labelled λO protein.

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